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Molecular Cloning of the Harvey Sarcoma Virus Closed Circular DNA Intermediates: Initial Structural and Biological Characterization

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Supercoiled Harvey sarcoma virus (Ha-SV) DNA was extracted from newly infected cells by the Hirt procedure, enriched by preparative agarose gel electrophoresis, and digested with *Eco*RI, which cleaved the viral DNA at a unique site. The linearized Ha-SV DNA was then inserted into λgtWES.λB at the *Eco*RI site and cloned in an approved EK2 host. Ha-SV DNA inserts from six independently derived recombinant clones have been analyzed by restriction endonuclease digestion, molecular hybridization, electron microscopy, and infectivity. Four of the Ha-SV DNA inserts were identical, contained about 6.0 kilobase pairs (kbp), and comigrated in agarose gels with the infectious, unintegrated, linear Ha-SV DNA. One insert was approximately 0.65 kbp smaller (5.35 kbp) and one was approximately 0.65 kbp larger (6.65 kbp) than the 6.0-kbp inserts. R-looping with Ha-SV RNA revealed that the small (5.35 kbp) insert contained one copy of the Ha-SV RNA. Preliminary restriction endonuclease digestion of the recombinant DNAs suggested that the middle-size inserts contained a 0.65-kbp tandem duplication of sequences present only once in the small-size insert; this duplication corresponded to the 0.65-kbp terminal duplication of the unintegrated linear Ha-SV DNA. The large-size insert apparently contained a tandem triplication of these terminally located sequences. DNA of all three sized inserts induced foci in NIH 3T3 cells, and focus-forming activity could be rescued from the transformed cells by superinfection with helper virus. Infectivity followed single-hit kinetics, suggesting that the foci were induced by a single molecule.

Studies of type C retrovirus replication have recently focused on the intracellular viral DNA species present after retroviral infection. Three major forms of viral DNA, all of which have been shown to be infectious, have been described: an unintegrated linear DNA intermediate, a covalently closed circular DNA intermediate, and the integrated viral DNA (3, 6, 7, 12, 13, 29, 30, 38, 39, 41). The unintegrated linear DNA has been reported to be a precursor for the circular form (26), but the immediate precursor of the integrated viral genome has not yet been established.

Recent reports of helper-independent avian retroviruses have revealed two interesting structural features of the unintegrated viral DNA (15, 25). First, the linear form of viral DNA was longer than that predicted from the size of genomic RNA (exclusive of the 3'-terminal polyadenylic acid). The termini of such DNA molecules contained the same sequences at both ends; they were arranged as direct terminal repeats containing sequences present at both the 5' and 3' ends of the viral RNA genome. Second, the supercoiled circular form of viral DNA consisted of at least two different-sized molecules. The larger corresponded to a molecule which would have been formed by joining the ends of the unintegrated linear viral DNA; it therefore contained the repeated sequences of the linear molecule as a direct tandem duplication. The smaller circular viral DNA was similar to the larger molecule, except that it contained only one copy of the sequences which were present twice in the larger circle. For the avian viruses, this duplication was estimated to be 0.3 to 0.35 kilobase pairs (kbp). The structure of the helper-independent murine viral DNAs has been studied less completely, but data for Moloney murine leukemia virus (Mo-MuLV) also suggested the existence of linear and circular forms analogous to the avian system, except that for Mo-MuLV

the duplication was shown to be approximately 0.6 kbp (8, 42).

The Harvey sarcoma virus (Ha-SV), which is a recombinant between Mo-MuLV and endogenous rat cell sequences (24, 28), is a replication-defective retrovirus whose unintegrated linear DNA has been shown to transform mouse cells (18). Recently, we have identified multiple Ha-SV circular DNA forms in acutely infected mouse cells (Chang et al., unpublished data). The structural and biological analysis of these molecules was limited, however, by the small amounts of viral DNA available and further complicated by the presence of helper virus DNA. The prospect of both purifying and amplifying these retroviral molecules by molecular cloning techniques, therefore, appeared especially attractive.

In this communication, we describe the isolation and preliminary characterization of the circular forms of Ha-SV DNA inserted in the λgtWES.λB vector and cloned in an approved EK2 host. We have obtained three unique size class λ.Ha-SV DNA molecules, two of which are analogous to previously described intermediates, but including a third that has not previously been identified in retrovirus infections. Each of three molecules induced transformation of NIH 3T3 mouse cells.

MATERIALS AND METHODS

Cells and viruses. The origin of NIH 3T3 mouse embryo fibroblasts has been previously described (16). The NIH 3T3 cell line chronically infected with Ha-SV and Mo-MuLV used as the source of virus for newly infected cells was also previously described (19, 20). Mo-MuLV titers were determined by the XC plaque test (23), and Ha-SV titers were determined by focus formation (16). The chronically infected cells yielded 10^6 to 10^7 PFU and focus-forming units per ml when titrated on the uninfected NIH 3T3 cells. The ratio of PFU to focus-forming units was about 2:1.

Two other chronically infected cell lines were used as a source of viral RNA for the synthesis of complementary DNA (cDNA) viral probes. The first was a Fisher rat embryo cell line chronically infected with Ha-SV and Mo-MuLV (28); the second was a mink lung cell line (ATCC CCL-64) chronically infected with Kirsten sarcoma virus and feline leukemia virus (28). The ratio of sarcoma viral RNA to helper viral RNA was >10:1 in both cell lines.

Cells were grown in Dulbecco-modified Eagle minimal essential medium (Flow Laboratories) with penicillin (100 U/ml) and streptomycin (100 μg/ml). This medium was supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories). The cultures were negative for mycoplasma by aerobic and anaerobic techniques (Flow Laboratories).

Restriction endonucleases. Restriction endonucleases were purchased from Bethesda Research Laboratories and New England Biolabs. Reaction conditions were those recommended by the supplier, except that usually 5 to 10 U of enzyme was used per μg of DNA. For the Hirt DNAs from mouse cells, uncleaved λ DNA was added to the reaction to monitor the completeness of the digestion.

Hybridization reagents and conditions. Single-stranded [^{32}P]cDNA probes were synthesized in a reaction catalyzed by avian myeloblastosis virus DNA polymerase and primed with oligomers of calf thymus DNA (35). Briefly, 10 μg of viral RNA isolated from virions purified from sucrose gradients was added to a 250-μl reaction consisting of 50 mM Tris-chloride (pH 8.0), 1 mM dithiothreitol, 6 mM $MgCl_2$, 50 mM NaCl, 180 μg of actinomycin D per ml, 1 mM each dGTP, dATP, and TTP, 0.2 mCi of [^{32}P]dCTP (350 Ci/mmol; New England Nuclear Corp.), 70 U of avian myeloblastosis virus polymerase, and 400 μg of calf thymus fragments per ml. After a 120-min incubation at 37°C, the preparation was extracted with an equal volume of 100 mM Tris-chloride (pH 8.0)-saturated phenol, chromatographed on a Sephadex G-75 column to remove unincorporated dCTP, alkali treated with 0.5 N NaOH at 37°C for 120 min, neutralized, and ethanol precipitated.

For hybridization of DNA on nitrocellulose filters, the filters were first pretreated for 30 min with 1% glycine in 4× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0), then for 2 h with Denhardt's buffer (5) (0.02% each bovine serum albumin, polyvynylpyrrolidone, and Ficoll) in 4× SSC, 1% glycine, and 50 μg of sonically treated salmon sperm DNA per ml, and then for a further 2 h in hybridization buffer consisting of Denhardt's buffer, 4× SSC, 0.1% sodium dodecyl sulfate, and 50 μg of salmon DNA per ml. The filters were then hybridized for 24 to 48 h in sealed plastic bags containing 1×10^6 to 2×10^6 cpm of [^{32}P]cDNA per ml in hybridization buffer. The above procedures were all carried out at 65°C. After drying, filters were exposed at −70°C to Kodak X-ray X-Omat film, using a Dupont Lightning Plus calcium tungstate intensifying screen (34). Similar results were obtained with the Ha-SV and Kirsten sarcoma virus cDNA probes, since both genomes share considerable homology with each other (24, 28).

Agarose gel electrophoresis and DNA transfer. Analytical electrophoresis of DNA samples was carried out in a horizontal gel apparatus (Bethesda Research Laboratories) in 1% agarose gels containing 20 mM sodium acetate, 40 mM Tris-chloride (pH 8.1), 2 mM EDTA, and 0.5 μg of ethidium bromide per ml (27). Bromophenol blue was added as a tracking dye, and samples were electrophoresed at 45 V for 16 h. Wild-type λ DNA cleaved with *Eco*RI or *Hin*dIII and labeled with ^{32}P was used as a marker in every gel.

DNA to be hybridized with [^{32}P]cDNA was transferred from the gel to nitrocellulose filters by the procedure of Southern (31) with minor modification. In brief, gels were alkali treated with 0.2 N NaOH and 0.6 M NaCl for 45 min and neutralized for at least 45 min with 1.0 M Tris-chloride (pH 7.5) and 0.6 M NaCl, and the DNA was transferred from the gel to the filter by capillary action for at least 16 h. The filters were then dried at 80°C in vacuo for 2 h.

Preparation of supercoiled Ha-SV DNA from newly infected cells. NIH 3T3 cells grown in roller bottles (Corning) were infected with media from cells

chronically infected with a mixture of Mo-MuLV and Ha-SV as previously described (18, 37). The multiplicity of infection was approximately 50. Sixteen hours after infection, the cells were fractionated by the Hirt procedure (14), and the DNA from the supernatant fraction was purified as previously described (18). Hirt supernatant DNA (15 mg) from 150 roller bottles was subjected to preparative electrophoresis in a 0.7% agarose gel as previously described (22), and 15-ml fractions were collected. Aliquots from each fraction were treated with 0.2 M NaOH at 37°C for 2 h, neutralized, and then trapped on nitrocellulose filters. The filters were then baked for 2 h at 80°C in vacuo and hybridized to a Kirsten sarcoma virus [^{32}P]cDNA probe. The covalently closed Ha-SV DNA (which migrated faster than linear Ha-SV DNA) was further identified by analytical agarose gel electrophoresis of representative fractions before and after digestion with *Eco*RI (which cleaves the Ha-SV DNA only once), transfer of DNA to nitrocellulose filters, hybridization with the [^{32}P]cDNA probe, and autoradiography. After pooling the fractions containing the closed circular Ha-SV DNA, residual agarose was dissolved in NaI, and the DNA was concentrated by adsorption to glass beads and eluted as previously described (40). The unintegrated linear Ha-SV DNA was also isolated in this manner. The circular DNA was then restricted with *Eco*RI, phenol extracted, and ethanol precipitated.

Bacteria and bacteriophage strains. The EK2-certified vector λgWES.λB and host *Escherichia coli* DP50 *supF* were generously supplied by David Tiemeier (17). *E. coli* strain LE392 was a gift from Lynn Enquist. *E. coli* N5428 [N100 (λA*am*$_{11}$ *b*$_2$ red$_3$ cI857 *Sam*7)] was kindly provided by Nat Sternberg, and *E. coli* gd805 [W3350 (λ*d gal-805* cI857 *Sam*7)] was received from Fred Blattner.

Bacterial media. TB broth contained 10 g of tryptone (Difco) and 5 g of NaCl per liter; the pH was adjusted to 7.2. TB broth was routinely supplemented with 10 mM $MgSO_4$ to enhance phage growth. For growth of LE392, TB broth was supplemented with 50 μg of thymidine per ml; for growth of DP50 *supF*, TB broth was supplemented with 100 μg of diaminopimelic acid and 50 μg of thymidine per ml. TB plates contained TB broth solidified with 1.2% agar (Difco) and were routinely supplemented with 10 mM $MgSO_4$, 50 μg of thymidine per ml, and 100 μg of diaminopimelic acid per ml. NZY broth contained, per liter, 10 g of NZ-amine A (Kraftco Corp., Lynnhurst, N.J.), 5 g of NaCl, 5 g of yeast extract (Difco), and 10 mM $MgCl_2$.

Preparation of phage DNA. Vector phage λgtWES.λB were propagated by lytic growth on *E. coli* LE392 in TB broth. After lysis, bacteria and debris were pelleted by centrifugation, the culture was adjusted to 1.0 M NaCl, and phage were precipitated by the addition of polyethyleneglycol to a 10% (wt/vol) concentration. The precipitate was collected by centrifugation and resuspended in 10 mM Tris-hydrochloride (pH 7.6)–10 mM $MgSO_4$. After adjusting this suspension to 3.82 M CsCl by the addition of solid CsCl, the solution was centrifuged to equilibrium, and the phage band was collected, diluted with 2 to 3 volumes of 3.82 M CsCl, and centrifuged again to equilibrium. After the second banding, the phage preparation was dialyzed and DNA was extracted as described by Sternberg et al. (32). DNA was prepared from amplified recombinant phage by the same technique, slightly modified for removal from the P4 facility (see below).

Purification of vector arms. After restriction of vector DNA with *Eco*RI restriction endonuclease, the DNA preparation (≥150 μg/ml) was adjusted to 2 M NaCl and annealed at 48°C for 12 to 20 h to form cohesive end dimers. The DNA preparation was layered on a 35-ml 10 to 40% sucrose gradient (1.0 M NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA), as described by Maniatis et al. (21), and centrifuged for 19 h at 16°C. The cohesive end dimers were collected, ethanol precipitated, redissolved in 10 mM Tris (pH 7.4)–1 mM EDTA at a DNA concentration ≥400 μg/ml, and used without further treatment for ligation and packaging.

Preparation of packaging extracts. The extracts for packaging DNA into infectious lambda particles were prepared and utilized as described by Blattner et al. (2) with only minor modification. The freeze-thaw lysate and sonic extract were each prepared from NS428 grown and induced in NZY medium as described by Blattner et al. Partially purified protein A was prepared from dg805 grown and induced in NZY medium also as described by Blattner et al. Whereas these authors call for the freeze-thaw lysate supernatant fraction to be freshly prepared on the day of use, we have found that this fraction can be prepared in bulk quantity, refrozen, and stored submerged in liquid N_2 for subsequent use without any significant loss in efficiency of packaging.

In vitro packaging. Purified *Eco*RI λgtWES.λB arms were mixed with *Eco*RI-restricted form I Ha-SV circular DNA, adjusted to a final concentration of 100 μg of vector DNA and 30 μg of Ha-SV DNA per ml, and ligated overnight in 12.5°C in the presence of 200 U of T4 DNA ligase per ml, 66 mM Tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 0.4 mM ATP. Ligated vector-substrate DNAs were then packaged in vitro into infectious lambda particles according to the protocol of Blattner et al. (2) without modification. The efficiency of the packaging reaction varied between 10^7 and 10^8 infectious particles per ml for native vector DNA and between 10^5 and 5×10^6 particles per ml for ligated vector arm-substrate mixtures.

Detection of specific recombinants. After the titer of recombinant phage in the packaging reaction had been determined, the complete packaging reaction was plated on DP50 *supF* at the appropriate dilution to give approximately six plaques per cm^2. After development of the recombinant plaques, nitrocellulose filters were applied to each dish, and phage particles from each plaque were transferred to the membranes by a modification (G. L. Hager and R. W. Wolford, manuscript in preparation) of the Benton and Davis (1) "plaque lift" technique that is designed to provide rapid and accurate location of specific recombinant plaques. DNA was denatured in situ on the membrane, immobilized by baking at 80°C in vacuo for 2 h, and hybridized to the Kirsten sarcoma virus [^{32}P]cDNA as described above, except that the hybridization was carried out at 42°C in 5× SSC with 50% formamide. Plaques that hybridized with the cDNA probe were

resuspended in 10 mM $MgSO_4$–10 mM Tris-hydrochloride (pH 7.4)–0.1% gelatin, and subcloned. The subclone plaques were transferred again to membranes and hybridized to confirm the identity of the recombinant. Independent subcloned recombinants were propagated on DP50 *supF* in TB broth, and recombinant DNA was prepared as described above.

Biological and physical containment. The EK2-certified vector λgtWES.λB was utilized in these experiments in conjunction with in vitro packaging under the conditions prescribed in the National Institutes of Health Guidelines for Recombinant DNA Research. All packaging reactions, plating of recombinant plaques, and propagation of recombinant phages was carried out in the P4 Mobile Containment Laboratory at the National Institutes of Health. DNA was prepared from recombinant phage within the P4 facility and carried through the preparation to the phenol extraction step. After the preparation was shown to be free of both viable bacteria and infectious bacteriophage, the DNA was removed from the facility for further characterization under P2 physical containment.

Infectivity studies. Transfections were carried out by a modification (33) of the calcium precipitation method (9, 10) as previously described (18). DNA isolated from NIH 3T3 cells or from calf thymus by the method of Gross-Bellard et al. (11) was used as carrier DNA at 25 μg/ml. Both carrier DNAs gave similar results.

The agarose gel electrophoresis infectivity study was performed with the same horizontal slab gel apparatus used for the biochemical studies. A 2-μg sample of *Eco*RI-digested λ. Ha-SV DNA was subjected to electrophoresis in a 1% agarose gel at 30 V for 72 h. *Eco*RI-digested, wild-type lambda DNA was coelectrophoresed in adjacent lanes as marker DNA. The location of the ethidium bromide band was noted for each insert, and a 5-mm slice containing the band was made for each insert. Because of the distance the inserts had been electrophoresed, the DNA from the minor bands would then be contained in other gel slices. The remainder of the gel from each lane was also sliced. The gel slices were then frozen and disrupted as previously described (6), and 20% of each slice was assayed for infectivity.

Electron microscopy. Heteroduplexes were formed by mixing equal amounts (5 to 10 μg) of each purified λ.Ha-SV DNA, alkali denaturing, and renaturing in 50% formamide–100 mM Tris-hydrochloride (pH 8.5)–1 mM EDTA for 2 h at 35°C. The DNA was mounted for microscopy by the formamide procedure essentially as described by Davis et al. (4).

R-loops (36) were formed by incubating a mixture of λ.Ha-SV DNA and Ha-SV RNA in a solution containing 70% formide, 0.1 M Tricine (pH 8.0), 0.25 M NaCl, and 0.01 M EDTA at 52°C for 2 h. This solution was spread with only the addition of cytochrome *c* (50 μg/ml) over a distilled-water hypophase, picked up on Parlodion-coated grids, stained with uranyl formate, and rotary shadowed with platinum-palladium. Grids were examined in a Siemens Elmiskop 101 at 40 kV accelerating voltage. Electron micrographs were taken on Kodak electron image plates at magnifications of 4,000 to 8,000. Magnification was calibrated with a grating replica (E. F. Fullam, no. 1000), and contour lengths were measured with a Numonics graphic calculator interfaced to a Wang 2200 computer.

RESULTS

Since it would be necessary to relate any cloned Ha-SV-specific DNA to a known Ha-SV genome, a provisional restriction endonuclease map was derived for the infectious, unintegrated, 6-kbp linear Ha-SV DNA (Fig. 1) found in the Hirt supernatant fraction from newly infected mouse cells (Chang et al., manuscript in preparation). The map was generated by digestion of the Hirt DNA with restriction endonucleases singly and in combination; the Ha-SV cleavage products were analyzed by agarose gel electrophoresis, transfer of the DNA to nitrocellulose filters (31), and hybridization with an Ha-SV [^{32}P]cDNA probe. The enzymes which cleaved the molecule at least once divided themselves into two groups: those which cleaved within 0.5 kpb of either end and those which did not recognize sites less than 0.8 kbp from the termini. It should be noted that the enzymes which form the first group (*Xba*I, *Sac*I, *Kpn*I, and *Sma*I) cleaved the molecule at both termini at sites less than 0.5 kbp from each end, the relative order of the restriction sites for these four enzymes was the same at both ends, and for each enzyme the sum of the two terminal cleavage products was about 0.6 to 0.65 kbp. No such symmetry was found for the other enzymes tested. These results, which are analogous to those reported for in vitro synthesized Mo-MuLV DNA (8), are compatible with the linear Ha-SV DNA containing a terminal repeat of about 0.6 kbp. (A similar physical map of the linear Ha-SV DNA genome has been drived by M. Goldfarb and R. A. Weinberg [personal communication].)

*Eco*RI cleaved linear Ha-SV DNA at a single site (Fig. 1). Since supercoiled Ha-SV DNA should also contain a unique *Eco*RI cleavage site, restriction of the circular viral DNA with this enzyme should generate a linear molecule of similar molecular size. Preliminary experi-

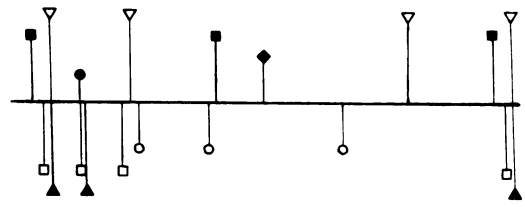

FIG. 1. *Restriction endonuclease map of 6-kbp unintegrated linear Ha-SV DNA isolated from infected mouse cells. The 5′ portion of the viral RNA is on the left; the 3′ portion of the viral RNA is on the right. Symbols:* (▽) *SmaI;* (■) *XbaI;* (◆) *EcoRI;* (●) *BamHI;* (○) *HindIII;* (□) *SacI;* (▲) *KpnI.*

ments fulfilled these predictions. Cleavage of supercoiled Ha-SV-specific DNA resulted in the generation of two major bands; the larger comigrated with the undigested linear Ha-SV DNA, whereas the smaller migrated as a linear molecule <1 kb shorter (data not shown). Although these data by themselves were compatible with the faster-moving component being derived either from a smaller circular molecule or from a molecule with an additional *Eco*RI site near the unique *Eco*RI site, further analysis of the circular molecules with other restriction enzymes eliminated this second possibility (Chang et al., manuscript in preparation). These results were therefore analogous to those previously reported for the two circular forms of Mo-MuLV (42).

Molecular cloning of supercoiled Ha-SV DNA. Because the Ha-SV circular DNA molecules in the Hirt supernatant DNA represented a very small fraction of the total DNA (estimated as 10^{-5} to 10^{-6}), several steps were undertaken to enrich for the viral DNA. First, the cells were infected at a high multiplicity of infection (about 50) with an Mo-MuLV pseudotype of Ha-SV. (Mo-MuLV was chosen as the helper virus because it rescues Ha-SV efficiently and its viral DNA is not cleaved by *Eco*RI [30]; therefore, the Mo-MuLV DNA would not be cloned along with the Ha-SV DNA.) Second, Hirt supernatant DNA from 150 newly infected roller bottles was pooled and subjected to preparative agarose gel electrophoresis. This procedure simultaneously enriched for the linear and circular Ha-SV DNA and permitted their separation by virtue of the faster migration rate of supercoiled DNA under the electrophoresis conditions used.

After pooling fractions containing circular Ha-SV DNA, the DNA was restricted with *Eco*RI, subjected to electrophoresis, and transferred to a nitrocellulose filter. Hybridization with the Ha-SV [^{32}P]cDNA resulted in three bands in the autoradiogram. In addition to the anticipated 6-kbp and faster-moving, Ha-SV-specific band noted above, a third band, which was <1 kbp larger than the 6-kbp viral DNA species, was also detected. In view of the presence of Mo-MuLV DNA in the gel fractions, it was not clear what this slower moving band represented, and because of the limited amount of Ha-SV circular DNA, investigation of this band was deferred.

The *Eco*RI-cleaved viral DNA was then ligated to the arms of the λgtWES.λB vector. These ligated DNA molecules were then packaged in vitro into infectious particles and amplified by lytic growth on the EK2 host. Approximately 3,000 plaques were obtained and screened for the presence of Ha-SV-specific DNA by the filter hybridization method of Benton and Davis (1). Seven recombinants were shown to contain Ha-SV-specific DNA upon subsequent subculturing. The DNA from six of these recombinant molecules was purified and subjected to analysis. In the experiments which follow, the structures of the λ.Ha-SV recombinant DNA molecules have been compared with that of the linear Ha-SV DNA. The theoretical structural relationship between the unintegrated linear Ha-SV DNA isolated from infected mouse cells, a circular fusion product of this linear DNA, and its permuted orientation when cloned in λ at the *Eco*RI site are shown diagrammatically in Fig. 2.

Heterogeneity of Ha-SV DNA in recombinant clones. The DNA from the six independently derived recombinant phage clones

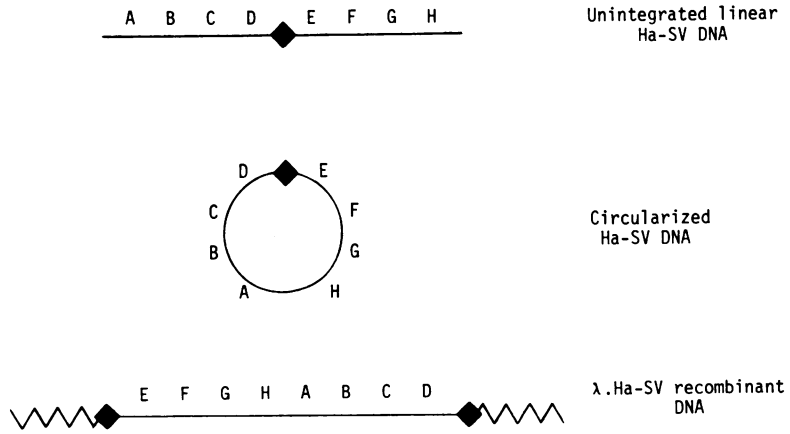

FIG. 2. *Schematic nucleotide sequence order of three molecular forms of Ha-SV DNA. The diamonds (◆) represent the EcoRI sites in Ha-SV and in λ.Ha-SV recombinant DNA. The diagonal lines (〰) represent the λ DNA arms (not drawn to scale). See text for details.*

was initially cleaved with *Eco*RI, subjected to agarose gel electrophoresis, and stained with eithidium bromide. Each of the recombinant DNAs contained the λgtWES.λB arms and a single DNA insert (Fig. 3A). The inserts were of three different sizes. Those in lanes 3, 4, 6, and 7 were apparently all the same size (about 6 kbp), and subsequent analysis has shown these four to be identical. The insert in lane 2 was <1 kbp larger and the insert in lane 5 was <1 kbp smaller than the other four DNA inserts. Based on their migration rate in gels and direct length measurements in the electron microscope (see below), the inserts and the recombinant phage from which they are derived have been designated as L (large), M (middle), and S (small), respectively.

To determine if the recombinant DNA preparations contained Ha-SV-specific DNA sequences, *Eco*RI-digested DNA was transferred from the gel to a nitrocellulose filter and hybridized with Ha-SV [^{32}P]cDNA. The autoradiogram shown in Fig. 3B indicated that each DNA insert reacted with the radiolabeled Ha-SV probe. Furthermore, the L (lane 2′) and M (lanes 3′, 4′, 6′, and 7′) inserts contained additional minor bands not visualized in the ethidium bromide-stained gel, whereas the S insert (lane 5′) consisted of a single band. The major band for every clone, however, corresponded to the insert seen in the ethidium bromide-stained gel, and the major band of the M inserts comigrated with the unintegrated linear Ha-SV DNA (lane 1′).

The presence of the minor bands in the M and L inserts represented an unanticipated finding whose meaning was initially unclear. In the autoradiogram, it can be noted that the minor bands were both larger and smaller than the major band of the insert. The smallest minor band of each clone comigrated with the single band of the small-size insert; the multiple bands detected in the five M and L clones comigrated with each other and appeared to represent a family of DNA inserts.

The three size classes of the Ha-SV inserts detected in the ethidium bromide-stained gel in Fig. 3A were confirmed by contour-length measurements of the DNA in the electron microscope. Their orientation with respect to the phage vector DNA was also determined by this procedure. Heteroduplex analysis of the six recombinant DNA preparations revealed that clones 1 (L), 2 (M), and 10 (S) contained inserts in the same orientation, whereas the inserts in clones 6, 8, and 12 (all M) were in the opposite orientation. The size of the inserts from clones 1, 2, and 10 were then derived from contour-length measurements made from *Eco*RI-cleaved molecules. The inserts were calculated to be 7.08, 6.22, and 5.52 kbp, respectively (Table 1).

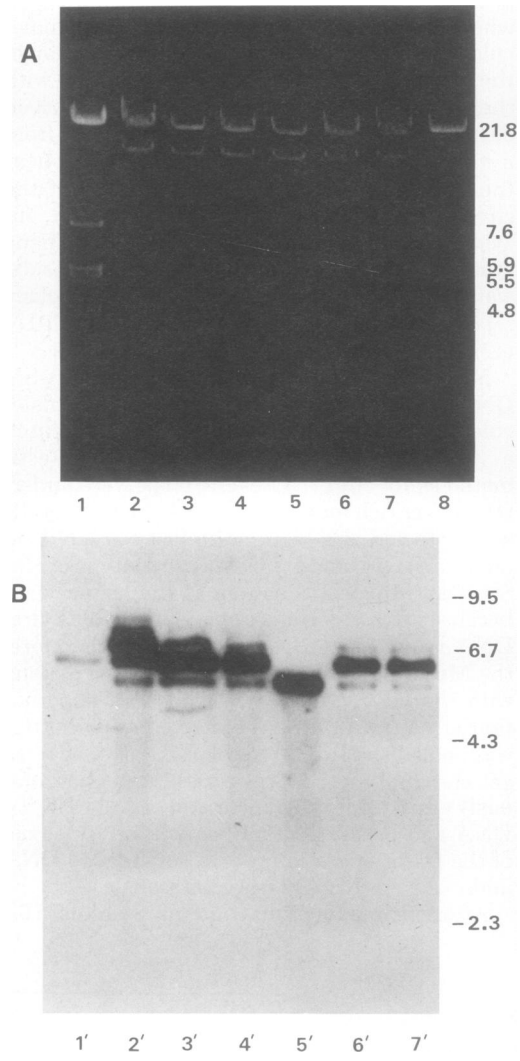

FIG. 3. *Agarose gel electrophoresis of EcoRI-digested recombinant DNAs. Samples, 1 μg, of DNA were subjected to electrophoresis, blotted onto nitrocellulose filters, and hybridized to the Ha-SV [^{32}P]cDNA, as described in the text. (A) UV fluorescence photograph of ethidium bromide-stained gel. (B) Blot analysis of gel similar to that in (A). (1, 8) EcoRI-digested, wild-type λ marker DNA; (2–7) λ.Ha-SV DNA clones 1, 2, 8, 10, 11, and 12, respectively; (1′) unintegrated Ha-SV linear DNA from infected mouse cells (not digested); (2′–7′) recombinant DNA clones 1, 2, 8, 10, 11, and 12, respectively. The numbers on the right side of (A) represent the length (in kilobase pairs) of the marker DNA fragments. The bars and accompanying numbers on the right side of (B) represent the position and size (in kilobase pairs) of HindIII wild-type λ fragments used as markers in this gel.*

TABLE 1. *Contour-length measurements of EcoRI cleavage products of λ.Ha-SV DNAs*

Clone	Contour length (μm)		
	λA fragment	Insert	λB fragment
1 (L)	6.90 ± 0.08 (14)[a]	2.27 ± 0.13 (63) 7.08 kbp[b]	4.55 ± 0.16 (23)
2 (M)	6.89 ± 0.10 (16)	1.95 ± 0.10 (62) 6.22 kbp	4.45 ± 0.38 (21)
10 (S)	6.84 ± 0.09 (17)	1.75 ± 0.08 (65) 5.52 kbp	4.50 ± 0.11 (20)

[a] Number in parentheses represents the number of molecules measured.

[b] Length measurements in kilobase pairs were derived by using the λB fragment as the internal standard and using 14.188 kbp as its length (L. Enquist, personal communication).

When these measurements were considered in conjunction with the restriction endonuclease data shown below, they were concluded to be compatible with the three size classes of inserts, differing from each other by approximately 0.65-kbp increments.

The source of the length differences between the three different-sized clones was determined by the formation of heteroduplexes between clones 1 and 2, 1 and 10, and 2 and 10. For each combination, only a single deletion loop was visualized. In the 25 to 30 molecules measured in each group, this loop was localized to approximately the same insert site for all three combinations, 0.58 genome length (standard deviation ± 1.5%) from one end of the heteroduplexed molecules. Heteroduplexes formed between the inserts after digestion with *Eco*RI further localized the site to 55% (±1%) from one end of the insert. Since each heteroduplex revealed only a single deletion loop, it was concluded that within the limits of the method, the M insert contained all of the sequences of the S insert plus an additional 0.65 kbp, the L insert contained the sequences present in the M insert plus 0.65 kbp, and the two 0.65-kbp sequences in the L insert not present in the small clone were tandemly arranged.

No visible deletion loops were observed when an individual DNA preparation was denatured and allowed to reanneal, despite scanning more than 100 molecules of each size class. This result indicated that the major bands seen in the gel analysis of the middle- and large-size recombinant clones represented the majority of the inserts.

Structure of Ha-SV inserts. The previous results showed that the inserts were closely related to each other and reacted to the Ha-SV [^{32}P]cDNA probe, but provided only incomplete information regarding their relationship to the Ha-SV genome, the structural relationship of the three different size inserts to each other, and the origin of the minor bands. The answers to these questions were sought by digestion of the recombinant clones with the restriction endonucleases used to generate the physical map of the linear Ha-SV DNA shown in Fig. 1 and also by heteroduplex analysis of the recombinant DNAs with Ha-SV genomic RNA under R-loop conditions. The fine-structure mapping of the cloned Ha-SV DNA will be presented elsewhere (Chan et al., manuscript in preparation). In this paper, the examination of the molecular structure of the cloned Ha-SV DNA has been confined to one restriction enzyme which does not cleave (*Hin*dIII) and one which does cleave (*Xba*I) within the putative terminal repeat sequences of the linear viral DNA.

The autoradiogram in Fig. 4 compares the hybridization of Ha-SV [^{32}P]cDNA to the *Eco*RI+*Hin*dIII digest of each of the three different-sized recombinant preparations and the unintegrated linear Ha-SV DNA obtained from infected mouse cells. Lane 7 shows the five fragments obtained after *Eco*RI+*Hin*dIII double digestion of the linear Ha-SV DNA. A circular DNA molecule formed by joining the termini of linear Ha-SV DNA would yield four fragments after *Eco*RI+*Hin*dIII digestion. Fragment C, D, and E cleavage products, which are the three internal fragments of the unintegrated linear Ha-SV DNA, would be derived from both molecular species; the circular form would generate a fusion product whose size was the sum of fragments A and B, which represent the end fragments of the linear viral DNA.

Several important features of the *Eco*RI+*Hin*dIII digests of the three different size recombinant clones (lanes 1 to 3) should be noted. First, the three different-sized inserts all contained four major fragments. The differences between the inserts were confined to the largest fragment, and the three smaller fragments comigrated with the three internal fragments of the Ha-SV linear DNA. Second, the major band of the large fragment of the M insert (lane 3, arrow) migrated at the position predicted for a molecule whose size was the sum of the A and B fragments of the *Eco*RI+*Hin*dIII digest of the Ha-SV linear DNA. Third, additional minor bands reminiscent of the bands observed after *Eco*RI digestion of the M and L inserts were seen adjacent to the large *Hin*dIII+*Eco*RI fragment of the M and L insert: none were associated with the large fragment derived from the S insert or the three smaller bands of any of the inserts. As in the *Eco*RI digest, the smallest minor band of both M and L inserts comigrated with the large fragment of the S insert. The intensity of the minor bands diminished with their distance from the major band, and the multiple bands

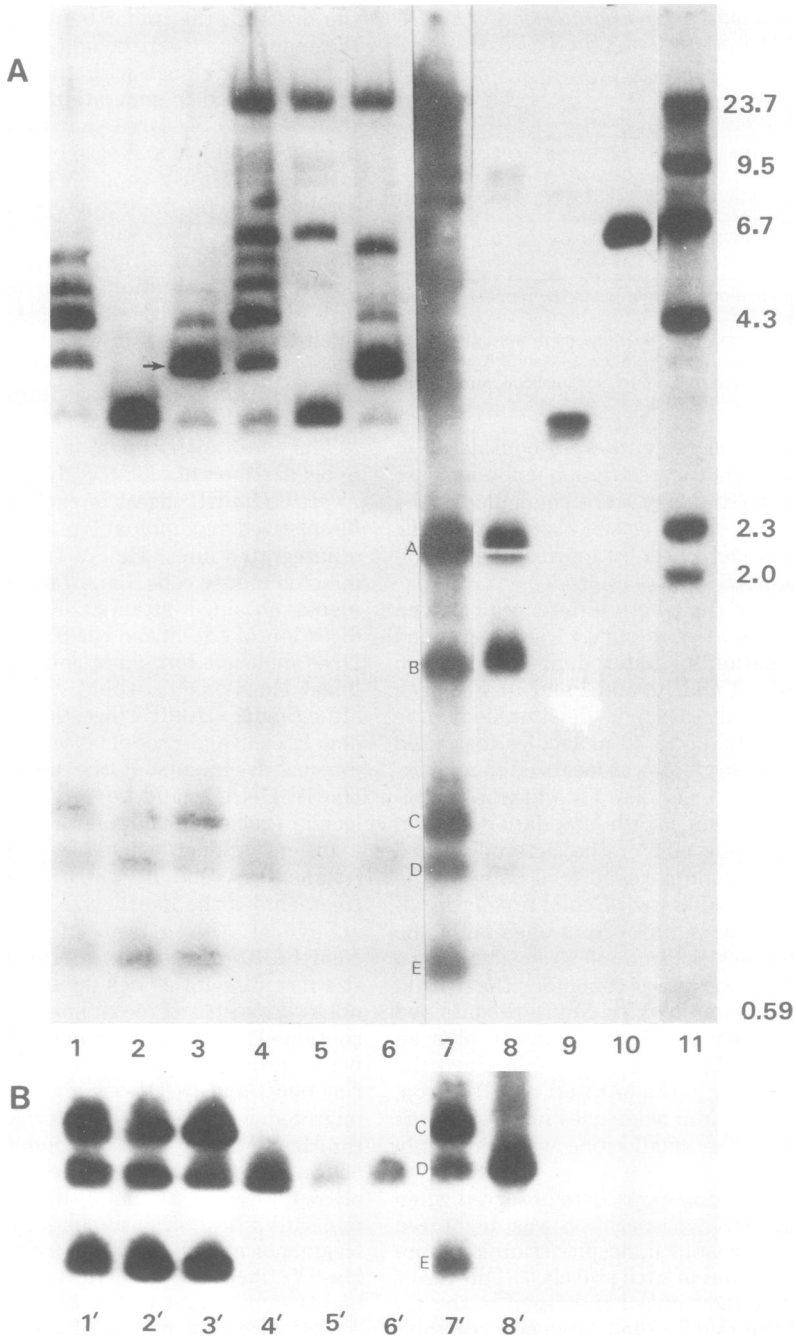

FIG. 4. *Southern blot analysis of recombinant DNAs and unintegrated linear Ha-SV DNA after digestion with HindIII+EcoRI. After digestion with EcoRI, HindIII, or HindIII+EcoRI, the DNAs were subjected to agarose gel electrophoresis and treated as described in Fig. 3. (B) Overexposure of the lower portion of (A) for lanes 1 to 8. (1–3) HindIII+EcoRI digests of λ.Ha-SV DNA clones 1 (L), 10 (S), and 12 (M), respectively; (4–6) HindIII digests of λ.Ha-SV DNA clones 1, 10, and 12; unintegrated linear Ha-SV DNA from mouse cells digested with (7) HindIII + EcoRI, (8) HindIII, and (9) EcoRI; (10) undigested linear Ha-SV DNA; (11) ^{32}P-labeled HindIII wild-type λ marker DNA. The numbers on the right side represent the length (in kilobase pairs) of the marker DNA fragments.*

comigrated with each other in approximately 0.65-kbp increments. These results indicated that the differences between the three different size inserts resided in the largest *Eco*RI+*Hin*dIII fragments (which represent the end fragments of the linear Ha-SV DNA) and suggested that the M insert contained all the sequences present in the Ha-SV linear DNA permuted in the order shown at the bottom of Fig. 2.

This interpretation of the data was confirmed and amplified when the recombinant DNA preparations were digested with *Xba*I (Fig. 5), an enzyme which cleaves within the terminal 0.6 kbp of each end of the Ha-SV linear DNA. Of the five fragments generated by digestion of the unintegrated linear Ha-SV DNA (lane 2) with *Eco*RI+*Xba*I (the D and E fragments were not resolved in this autoradiogram), the three internal fragments (A, B, and C) of the unintegrated linear Ha-SV DNA were also detected in the three different species of cloned Ha-SV DNA (lanes 6 to 8). The two 0.3-kbp end fragments in linear Ha-SV DNA have been replaced by a 0.65-kbp fragment in the M (lane 6) and L (lane 8) clones. This fragment was not present in the S clone. In addition, there were no minor bands around any of the fragments. These results again suggested that the M insert contained all of the sequences present in the linear Ha-SV DNA and included a tandem duplication of approximately 0.65 kbp. When combined with the results of the heteroduplex analysis of the recombinant DNA preparations, the data were also compatible with the S insert containing the sequences in the linear Ha-SV DNA except for the terminal repeat and the large insert containing a triplication of the 0.65-kbp region. The disappearance of the minor bands suggested that they, too, were associated with the 0.65-kbp repeat region and represented the acquisition and loss of copies of these DNA sequences.

The homology between the recombinant DNA preparations and the Ha-SV genome was also compared directly by hybridization of the S clone to the Ha-SV RNA genome under R-loop conditions (36). An electron micrograph of a molecule formed by this procedure showed a large loop of displaced single-stranded DNA and a corresponding region of an RNA-DNA hybrid (Fig. 6). A small projection of single-stranded RNA could be detected near the middle of the RNA-DNA hybrid. Contour-length measurements of the circular RNA-DNA hybrid region of 35 intact molecules gave a mean value of 1.74 ± 0.04 μm (5.4 kbp). Cleavage of the recombinant clone with *Eco*RI before hybridization produced circular, duplex molecules without any visible internal structure other than a short, presumably polyadenylated projection. These circular hybrids (25 molecules) measured 1.77 ± 0.07 μm in length, a value similar to that of the Ha-SV insert and the Ha-SV RNA. No circles were

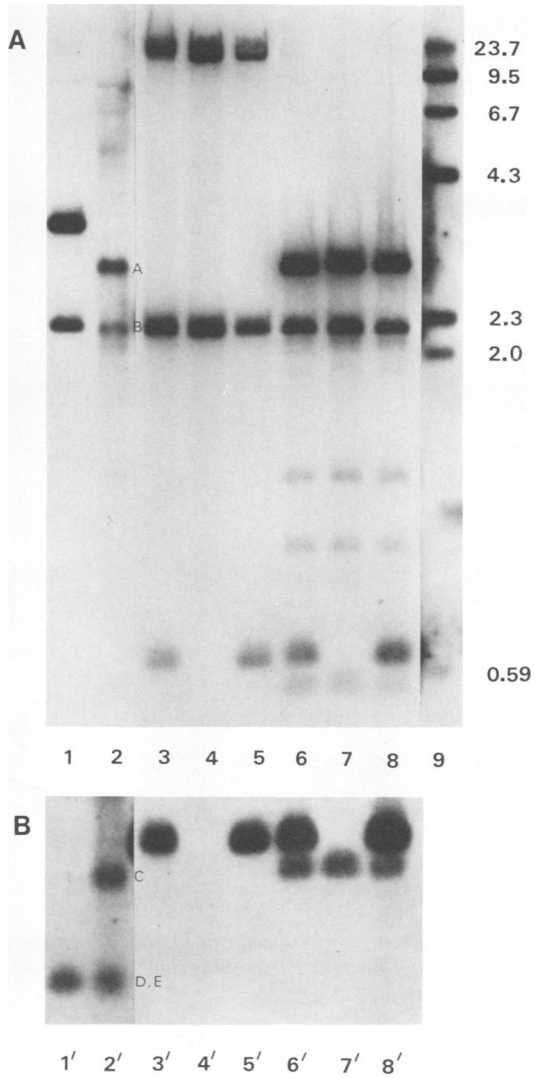

FIG. 5. *Southern blot analysis of recombinant DNAs and unintegrated linear Ha-SV DNA after digestion with XbaI+EcoRI. DNAs were digested with XbaI, EcoRI, or XbaI+EcoRI, subjected to agarose gel electrophoresis, and treated as described in Fig. 3. (B) Overexposure of the lower portion of (A) for lanes 1 to 8. Unintegrated linear Ha-SV DNA digested with (1) XbaI, (2) XbaI+EcoRI; (3-5) XbaI digest of λ.Ha-SV DNA clones 12 (M), 10 (S), 1 (L); (6-8) XbaI+EcoRI digest of λ.Ha-SV DNA clones 12, 10, and 1; (9) ^{32}P-labeled, HindIII-digested, wild-type λ marker DNA.*

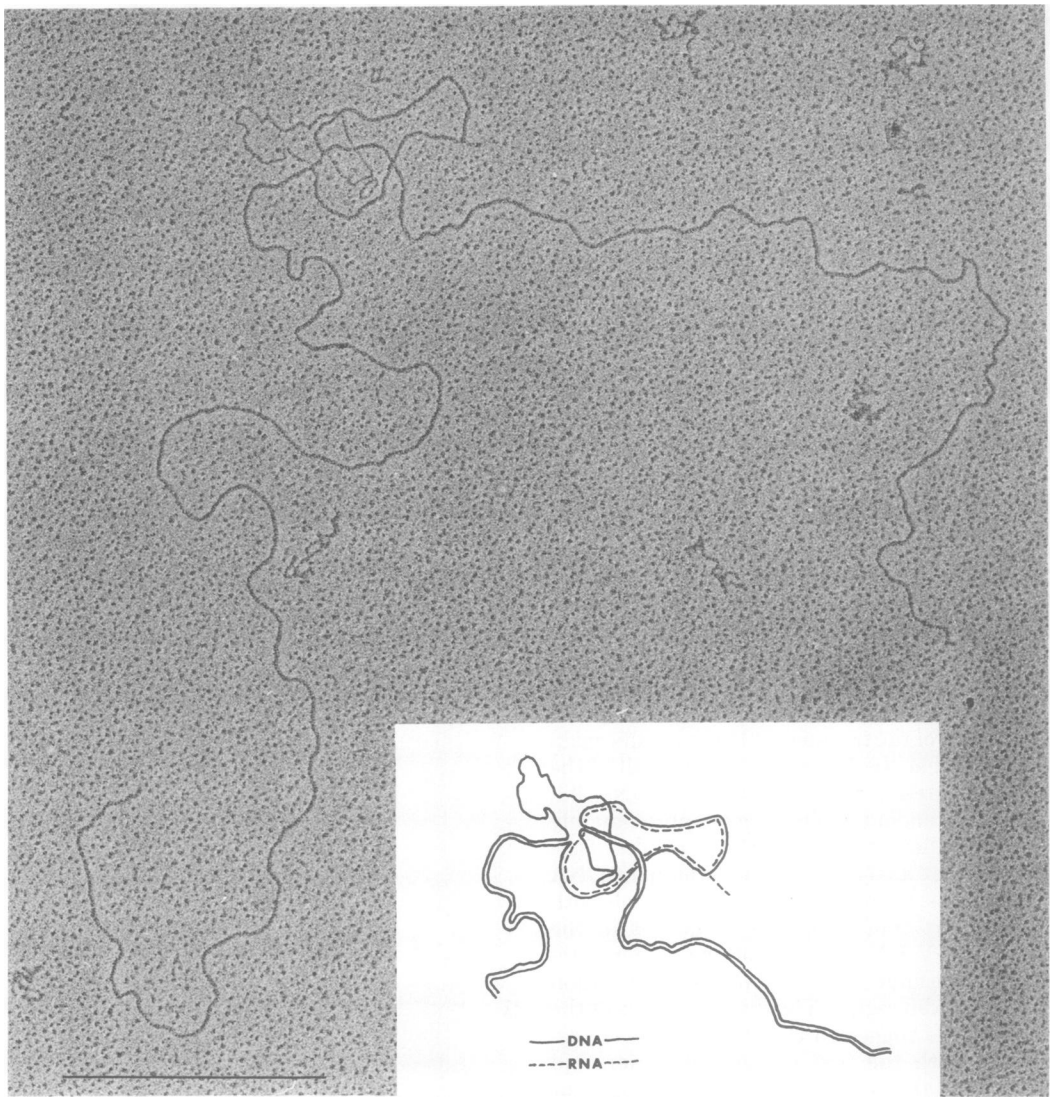

FIG. 6. *Electron micrograph of hybrid formed between Ha-SV RNA and a recombinant DNA molecule (clone 10). An illustrative tracing is shown in the inset. Unhybridized, collapsed RNA molecules are visible in the field. Bar, 1 μm.*

formed without the addition of RNA under these conditions.

Although not all molecules had an identifiable RNA tail as seen in Fig. 6, the distance of the tail from one end of the RNA-DNA hybrid was analyzed for 25 molecules and was mapped 55.7 ± 0.006% from one end of the recombinant molecule. This single-stranded structure most likely represents one end of the viral RNA, presumably the polyadenylated tail at the 3′ end of the RNA molecule, since it is devoid of secondary structure and the length of the RNA-DNA hybrid is equivalent to the length of the entire Ha-SV RNA genome exclusive of polyadenylation. These results show directly that the Ha-SV recombinant DNA is colinear with the Ha-SV RNA genome and that sequences present at or near the ends of the RNA genome are joined near the middle of the recombinant DNA molecule.

Infectivity of Ha-SV DNA inserts. The foregoing studies suggested that the complete genetic information present in the Ha-SV RNA was present in all three size classes of the Ha-SV DNA preparations. It was therefore of considerable interest to determine if the cloned viral

DNA might be infectious for eucaryotic cells. Closed circular Mo-MuLV DNA had previously been shown to be infectious for mouse cells (29); however, since the circular DNA used in those experiments had not been fractionated according to size, the structure of the molecule or molecules which gave rise to infection was not established.

After digestion with *Eco*RI, DNA from each of the different-sized inserts was transfected onto NIH 3T3 cells, a line which had previously been shown could be nonproductively transformed by linear Ha-SV DNA (18). Foci of transformed cells began to appear 5 to 7 days after transfection with each of the Ha-SV DNAs. These foci from the three different-sized inserts were morphologically indistinguishable. There were, however, late appearing (11 to 13 days after transfection), poorly defined areas of apparently transformed cells after transfection with the L insert which were noted only rarely with the M and S inserts. It is not yet clear if these latter areas represent primary or secondary foci. At 10 days after transfection, the specific infectivity (foci per microgram of DNA) of the three different-sized inserts were quite similar, but the cloned DNA preparations were about one order of magnitude less infectious than the unintegrated linear Ha-SV DNA derived from mouse cells. After superinfection with Mo-MuLV as a helper virus, Ha-SV could be rescued with high efficiency ($>10^6$ focus-forming units/ml) from cultures containing multiple foci induced by any of the three size inserts, which suggested that the complete Ha-SV genome was present in at least some of the foci.

In view of the multiple Ha-SV-specific bands seen on filter hybridization of the L and M inserts, it was important to determine which molecular species was inducing the foci in clones of each size. DNA from each size clone was therefore restricted with *Eco*RI and subjected to agarose gel electrophoresis. The gels were sliced, and the DNA from the slices was assayed for infectivity. Each size insert displayed a sharp peak of infectivity which correlated with the ethidium bromide band of that insert (Fig. 7). This result indicated that after *Eco*RI digestion, the infectivity of each Ha-SV recombinant DNA was due principally to the predominant Ha-SV insert of that clone. In addition, these results formally ruled out the possibility that the infectivity of the DNAs might be due to other minor species of DNA not even detected in the filter hybridization experiments.

Although these studies indicated that molecules of a particular size were responsible for the transformed foci, they did not indicate whether each focus was the result of the interaction of more than one of these molecules or was the result of a single transfecting molecule. This question was particularly pertinent because of the permuted form of the Ha-SV recombinant DNA molecules; intracellular end-to-end ligation of two insert molecules at the *Eco*RI sites would, in the proper orientation, generate a mol-

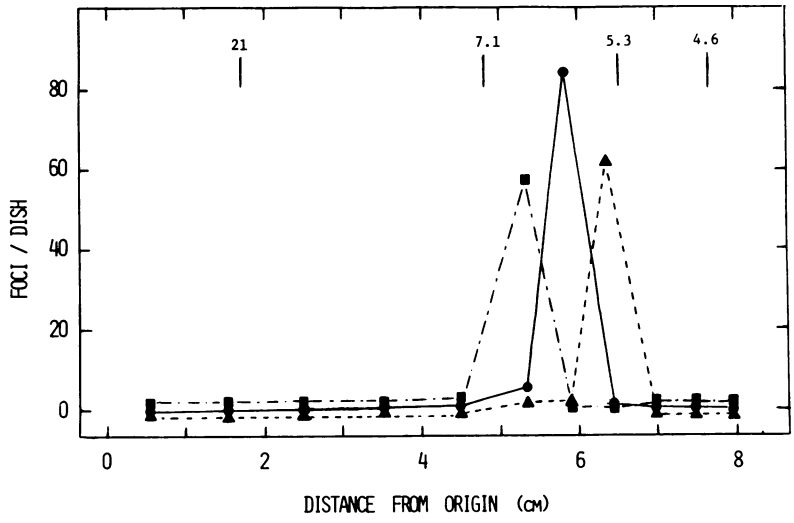

FIG. 7. *Infectivity of agarose gel electrophoresis of EcoRI-digested λ.Ha-SV DNAs. DNAs were subjected to electrophoresis (from left to right), and the infectivity of the gel fractions was assayed as described in the text. The vertical bars and the numbers above them indicate the location and length (in kilobase pairs), respectively, of EcoRI-digested wild-type λ marker DNA run in an adjacent lane. Symbols: (■) clone 1 (L); (●) clone 2 (M); (▲) clone 10 (S).*

ecule which internally was not colinear with the unintegrated linear Ha-SV DNA. In a titration assay, foci induced by this latter mechanism would fall off with two-hit kinetics, whereas foci induced by a single molecule would follow a single-hit titration pattern.

In Fig. 8 are presented the results from two separate experiments of the titration of foci generated from the inserts after *Eco*RI digestion. The foci induced from each insert followed a single-hit titration pattern with close to single-hit kinetics. These results imply that the majority of the foci resulted from a single transfecting molecule, although they do not rule out the possibility that a small minority of foci resulted from the joining of two transfecting molecules.

DISCUSSION

In this initial effort at molecular cloning of a retroviral genome from in vivo infection, the closed circular DNA intermediates of the Ha-SV genome were chosen because they were potentially interesting structurally and functionally, DNA could be enriched for the circular forms, and the unique *Eco*RI cleavage site in Ha-SV DNA made it possible to clone the entire Ha-SV circular molecule, since the certified lambda vector λgtWES.λB possesses two *Eco*RI sites, making it a suitable cloning vehicle (17). It was estimated that preparative agarose gel electrophoresis of the circular Ha-SV DNA followed by *Eco*RI digestion of the fractions containing the circular molecules would, when combined with the contraints of recombinant molecules which register as plaques in the λgtWES.λB system, result in about a 10^3-fold enrichment for the Ha-SV DNA. Instead of representing one molecule in 10^5 to 10^6 molecules of cellular DNA, the Ha-SV DNA would represent one molecule in 10^2 to 10^3 of the recombinant DNAs. The availability

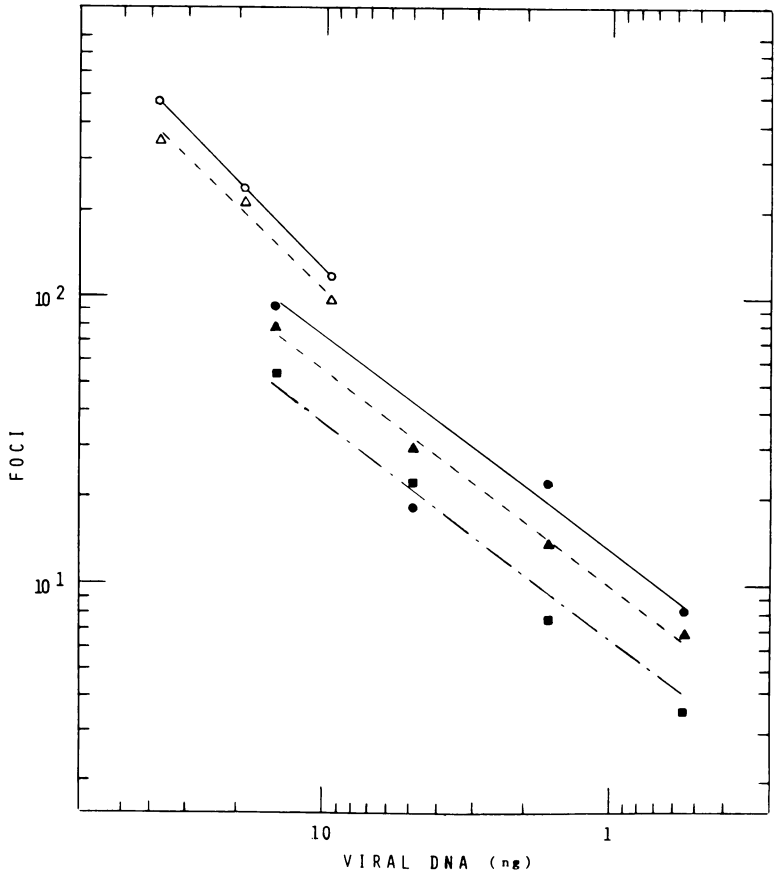

FIG. 8. *Titration of focus-forming activity of EcoRI-digested λ.Ha-SV DNAs. EcoRI-digested DNAs were assayed for focus formation as described in the text. The horizontal axis indicates the amount of Ha-SV DNA insert transfected. Results from two separate experiments are shown. The open and closed symbols are from experiments 1 and 2, respectively. Symbols:* (■) *clone 1 (L);* (○, ●) *clone 2 (M);* (△, ▲) *clone 10 (S).*

of the in situ assay to screen large numbers of plaques for those containing Ha-SV-specific DNA made it technically feasible to screen several thousand plaques in the P4 containment facility. In the data obtained here, 7 of about 3,000 putative recombinant molecules (about one in $10^{2.6}$ molecules) were Ha-SV specific.

Several points can be made from our initial analysis of the structure and biological activity of the Ha-SV recombinant clones. Restriction endonuclease and electron microscopic data indicate that three different-sized Ha-SV DNA molecules have been isolated. All three size classes of inserts apparently contain at least one copy of the sequences present in Ha-SV genomic RNA. It has been shown in a preliminary manner in this paper, and will be documented more completely in a subsequent study (Chan et al., manuscript in preparation), that these three molecules differ from each other by the number of copies they contain of a particular 0.65-kbp set of sequences. The S insert contains only one copy of these sequences and the M insert contains two copies, whereas the L insert contains three copies of the 0.65-kbp sequence. The data suggest that the repetitions represent tandem repeats.

The presence of the minor bands in the M and L inserts after digestion with two restriction endocucleases (*Eco*RI and *Hin*dIII) which did not cleave in the repeat region was a striking feature of the data reported here. Since these minor bands were not seen after digestion with *Xba*I, which apparently cleaved the Ha-SV DNA in the 0.65-kbp repeat sequences, it follows that the minor bands may be accounted for by acquisition and loss of complete copies of the 0.65-kbp sequence. This phenomenon will be documented and discussed more completely elsewhere (Chan et al., manuscript in preparation), but it may be noted here that the results suggest that the change in the number of copies of the repeat sequence occurred during amplification of the recombinant clones in the Rec^+ procaryotic host.

The middle-size insert apparently has the structure of the unintegrated linear Ha-SV DNA joined at its ends and then linearized at its unique *Eco*RI site. This M insert corresponds to the larger of the two retrovirus circular forms previously reported for helper-independent viral genomes (15, 25, 42), whereas the S corresponds to the smaller circular form. The L insert, which is the molecule with the triplication, has not previously been described in eucaryotic cells. Since copies of the repeat sequence may be acquired during amplification of the middle-size clone in the procaryotic host, as noted above, it is formally possible that the large insert might have arisen as a cloning artifact. However, this hypothesis is unlikely to be correct because the rate of acquisition of a new copy of the repeat sequence during replication of the λ.Ha-SV DNA must be quite low, since no deletion loops were seen when an M clone was denatured and allowed to reanneal to itself. On the other hand, it is likely that the triplicated circular form may exist in eucaryotic cells because preliminary restriction endonuclease data from closed circular Ha-SV DNA obtained from mouse cells infected at a high multiplicity of infection are consistent with this hypothesis (Chang et al., unpublished data). Since acquisition and loss of copies of the repeat sequence may occur during replication of the M and L clones in the procaryotic host, it should not be entirely unexpected if the same phenomenon is seen in the eucaryotic cell, especially at the high multiplicity of infection used in the experiments reported here.

The availability of the cloned Ha-SV DNA provided an initial opportunity to compare the infectivity of DNA with one, two, or three copies of the repeat sequence. The infectivity studies showed first that after digestion with *Eco*RI, transfection with all three sizes of λ.Ha-SV DNA onto NIH 3T3 cells induced focus formation with similar efficiency and that focus-forming virus could be efficiently rescued from mass cultures when superinfected by a helper virus. These results strongly suggest that at least some foci contained the complete Ha-SV genome. However, these experiments did not determine if focus-forming virus can be rescued with high efficiency from all foci, since the superinfection was carried out with cultures containing multiple foci.

The infectivity of DNA from the agarose gel electrophoresis showed that after *Eco*RI digestion more than 90% of the foci generated from each clone came from the major band; this result showed directly that all three size inserts were infectious. A single molecule was capable of inducing a focus, since focus formation followed a single-hit titration pattern. Therefore, molecules with and without a repetition of the 0.65-kbp sequence can have similar degrees of infectivity.

Molecular cloning of retroviral DNAs offers the prospect of being able to study their structural organization in great detail as well as providing reagents for probing cells with defined segments of these genomes. Since the cloned Ha-SV DNA molecules retained their biological activity when inoculated into susceptible eucaryotic cells, this result offers the prospect of studying the functional activity of these molecularly cloned molecules after they have been

perturbed in defined ways before inoculation into the eucaryotic cells. In this manner, it should be possible to correlate the structural features of the retroviral genome which account for their phenotypic characteristics.

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